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Research Article

Detection, isolation, and characterization of the cucumber mosaic virus in *Pseudostellaria heterophylla* from Korea

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Abstract Weeds play an important role in the survival of viruses and are potential inoculum sources of viral diseases for crop plants. In this study, specimens of *Pseudostellaria heterophylla* exhibiting symptoms of the *cucumber mosaic virus* (CMV) were collected in Bonghwa, Korea. The characteristics of the disease were described and leaf RNA was extracted and sequenced to identify the virus. Three CMV contigs were obtained and PCR was performed using specific primer pairs. RNA from positive samples exhibiting CMV leaf symptoms was amplified to determine the coat protein. A sequence comparison of the coat protein gene from the CMV BH isolate shared the highest nucleotide identity (99.2%) with the CMV ZM isolate. Phylogenetic analysis showed that CMV-BH belonged to subgroup IA and that the most closely-related isolate was CMV-ZM. All test plants used for the biological assay were successfully infected with CMV and exhibited CMV disease symptoms such as blistering, mosaic, and vein yellowing. To our knowledge, this is the first report of CMV infection in *P. heterophylla* from Korea.

Keywords Bioassay, *Cucumber mosaic virus*, *Pseudostellaria heterophylla*, RNA sequencing

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Introduction

Pseudostellaria heterophylla (Caryophyllaceae) is a herbaceous perennial widely distributed throughout the mountains in Korea. Indeed, the species is found in Asia, Europe, and North America. It has long been used in traditional Chinese medicine for treatment of chronic diseases (Hu et al. 2019; Ma et al. 2011), and taxonomic research has documented eight species of *Pseudostellaria*, three of which, *P. heterophylla*, *P. palibiniana*, and *P. longipedicellata*, are common in Korea (Jo et al. 2014; Lee 1989; Lee et al. 2012).

Pseudostellaria heterophylla can be easily infected with viruses that cause symptoms such as mosaic, crinkling, and stunted growth; all leading to biomass reduction. Four viruses (*Broad bean wilt virus, cucumber mosaic virus* (CMV)*, Tobacco mosaic virus, and Turnip mosaic virus*) have been reported in *P. heterophylla* in China, whereas no studies in this field have been reported in Korea (Kuang et al. 2017; Shong and Pu 1991). Among these viruses, CMV (*Cucumovirus* genus in the family *Bromoviridae*) was first described in 1916, and found distributed worldwide (Doolittle 1916). The viral genome of CMV is composed of a positive sense, single-stranded RNA molecule divided into three segments; in all, this genome encodes five proteins (1a, 2a, 2b, 3a, and 3b). RNA1 is monocistronic, encoding only protein 1a, while RNA2 and RNA3 are dicistronic, as they encode proteins 2a and 2b, and 3a and 3b, respectively (Roossinck 1999). CMV has an extensive host range, infecting approximately 1,000 species in 100 families including main crops, fruits, and ornamentals, whereby it is surely one of the economically most important plant viruses in the world. As for transmission, in addition to being seed-borne, CMV is readily transmitted mechanically and by insect vectors (Ali and Kobayashi 2010; Jalender et al. 2017; Mauck et al. 2010). Further, CMV is transmitted by more than 75 aphid species in a non-persistent fashion (Palukaitis et al. 1992).

Weed hosts play an important role in the spread and as a primary source of inoculum of viral diseases because they keep the virus alive during the fallow season (Abdullahi et al. 2001; Orosz et al. 2017). In addition, weed hosts influence the spread of the virus because they have an extensive and intimate relationship with the life cycles of insect vectors (Bitterlich and MacDonald 1993). Nevertheless, studies on forest weed hosts in Korea are very scarce. Therefore, the main objective of this research was to identify CMV in a forest weed host, and to provide basic information on weed virus disease in forest environments in Korea.

Materials and Methods

Sample collection

In May 2019, three *P. heterophylla* specimens with mosaic symptoms on the leaves were collected in Bonghwa-gun, Gyeongbuk Province of Korea. Two of these samples were suspected of viral infection and one was asymptomatic (Fig. 1). Plant leaves were cut, placed in zipper bags, and divided into two groups. One group consisted in individual leaf samples and the other group consisted in a mixture of 100 mg of each of the three plants collected. Samples were ground with mortar and pestle under liquid nitrogen and stored until use at -80°C in a deep freezer.

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from leaf samples using two commercial total RNA extraction kits. Total RNA from the individual leaf samples of the three plants collected was extracted with the easy-spin Total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) according to instructions by the manufacturer. Extracted RNA from individual samples was used as template for polymerase chain reaction (PCR). First strand complementary DNA (Fs-cDNA) was synthesized from 3 μL of total RNA with RN25 primer in a 40 μL reaction using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

RNA sequencing and analysis

Total RNA from mixed samples was extracted using the Maxwell® RSC plant RNA kit (Promega Corporation, Madison, WI, USA) and DNA contamination was eliminated using DNase. Ribosomal RNA was removed with TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA, USA) and randomly truncated to fragment purified RNA prior to adapting and sequencing. Reverse transcription of fragmented RNA into cDNA library construction and adapter ligation was performed with a TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). RNA sequencing was performed in an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA) with paired-end 101 bp reads. Quality assessment of the sequenced raw reads was performed using FastQC v0.11.7. Adapter sequence trimming and data filtering was performed using Trimmomatic 0.38, and trimmed reads were used to perform *de novo* transcriptome assemble using the Trinity program (version: trinityrnaseq-r20140717). Transcripts were searched against NCBI Nucleotide (nt) (ver. 20180116) and NCBI non-redundant Protein (NR) (ver. 20180503) using BLASTN of NCBI BLAST and BLASTX of DIAMOND software with an E-value default cutoff of 1.0E -5. All processes were performed by the public biotechnology company Macrogen Co. (Seoul, Korea).

PCR amplification and sequence analysis

Based on RNA sequencing information, PCR amplification

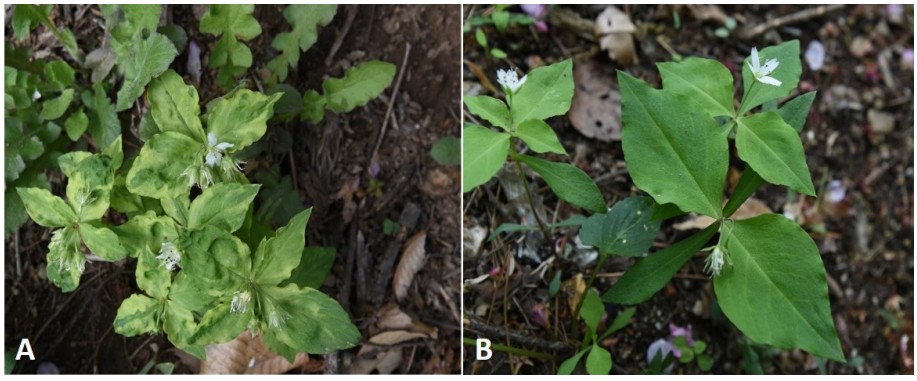

Fig. 1 Disease symptoms displayed by the leaves of *Pseudostellaria heterophylla*: (A) mosaic symptoms and (B) symptomless leaves

was performed using 2X TOPsimple DyeMIX (aliquot)-HOT premix (Enzynomics, Daejeon, Korea). For confirmation of viral infection, CMV specific pairs were used as described by Kwon et al. (2018). To determine the complete coat protein (CP) gene, specific primer pairs (CMV-CP-F: GCA ATC GGG AGT TCT TCC GCG / CMV-CP-R: GGA TGG ACA ACC CGT TCA CC 1138) were used. The PCR reaction mixture was contained in a 40 μL final volume, 20 μL of premix, 1 μL of specific primer sets, 1 μL of cDNA, and 17 μL sterile distilled water. PCR was carried out according to the following program: initial denaturation at 95°C for 10 min, 30 cycles of denaturation at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min 30 s; and a final extension at 72°C for 5 min. The amplified products were electrophoresed on a 1% agarose gel and stained with EcoDye DNA Stain (SolGent, Daejeon, Korea) to confirm in TAE buffer. The positive target bands were cleaned up using the Expin PCR SV kit (GeneAll, Seoul, Korea). The purified DNA sequence was determined directly using the Sanger sequencing method with specific primer pairs. Phylogenetic tree analysis and nucleotide comparison was performed to confirm the subgroup by comparison with 17 previously reported CMV isolates using the DNAMAN software package (ver. 5.2.10, Lynnon Biosoft, Canada) (Kim et al. 2014). CMV isolate sequences were collected from NCBI GenBank.

Isolation and pathogenicity test

To test the pathogenicity of the virus infecting *Pseudostellaria heterophylla*, mechanical inoculation was performed using leaf sap. Sap from symptomatic leaves was extracted in 0.01 M potassium phosphate buffer (pH 7.0) using mortar and pestle. Mechanical sap inoculation was carried out using silicon carbide (-400 mesh) by rubbing onto indicator plants at the 3 to 5 leaf stage. Indicator plants used included 22 species from four families: Cucurbitaceae, Leguminosae, Solanaceae, and Chenopodiaceae. Indicator plants were maintained in a plant growth chamber at 25°C for 6 weeks. The inoculated indicator plants were checked daily for symptom outbreak. Viral presence was finally confirmed by reverse transcription (RT)-PCR.

Results

Results of RNA sequencing

Total RNA extracted of mixed leaf samples was used for RNA sequencing and analysis to identify the virus(es) responsible for foliar mosaic symptoms in *Pseudostellaria heterophylla*. The number of total reads from raw data obtained was 124,988,440; GC content was 46.41%, and the Phred quality score ≥30 (Q30) was 95.99%. All 345,554 contigs were confirmed after *de novo* transcriptome assembly. Of these, three large contigs were confirmed which represented nearly the complete genome of CMV RNA 1, 2, 3 (Fig. 2, Table 1). One contig (3269 nt) shared the highest nucleotide identity (94.62%) with CMV RNA1 RP20 isolate from pepper in Korea (GenBank accession no. KC527794). The second contig (2950 nt) shared the highest nucleotide identity (98.07%) with CMV RNA2 KoPF isolate from passion fruit in Korea (KR535606). The third contig (2179 nt) shared the highest nucleotide identity (98.21%) with CMV RNA3 Rs strain from *Raphanus sativus* (AJ517802).

Detection and isolation of CMV in *Pseudostellaria heterophylla*

Results of PCR of total RNA extracted from individual samples showed that two symptomatic samples were positive to CMV, while an asymptomatic sample was negative. PCR products of the 657 bp (expected size) obtained, were directly sequenced. The confirmed sequences were analyzed using NCBI BLASTN and revealed 92% to 99% identity with previously reported CMV isolates; this positive sample was designated as CMV-BH isolate.

A bioassay was carried out using 22 indicator plant species to isolate and confirm the symptoms induced by CMV-BH. The CMV-BH isolate caused necrotic local lesions on leaves of *Citrullus vulgaris*, *Cucumis sativus*, *C. melo*, *Vigna sinensis*, *Pisum sativum*, *Nicotiana occidentalis*, and *Chenopodium amaranticolor*, 3 to 5 days post inoculation (dpi) (Fig. 3A, B, C). Chlorotic local lesions developed on leaves of *Cucurbita pepo*. Among cucurbitaceous indicators, *C. sativus*, *C. pepo*, and *C. melo* all developed chlorotic spots, vein yellowing, and mosaic symptoms on the upper leaves at 7 to 10 dpi (Fig. 3D, E). However, *C. vulgaris* showed no symptoms. As for *leguminous* indicators, *Phaseolus angularis* and *P. vulgaris* showed mosaic and mottle symptoms, while *Glycine max* showed no symptoms on the upper leaves at 14 dpi. In turn, within solanaceous indicators, blistering and/or mosaic mainly appeared at 7 to 14 dpi on the upper leaves of *Lycopersicon esculentum*, *N. benthamiana*, *N. debney*, *N. glutinosa*, *N. occidentalis*, *N. rustica*, *N. tabacum* var. KY57, *N. tabacum* var. Samsun, *N. tabacum* var. Xanthi nc, *N. tabacum* var. Turkish, *Physalis floridana*, and *Solanum melongena* (Fig. 3F, G, H, I). *Chenopodium amaranticolor* did not

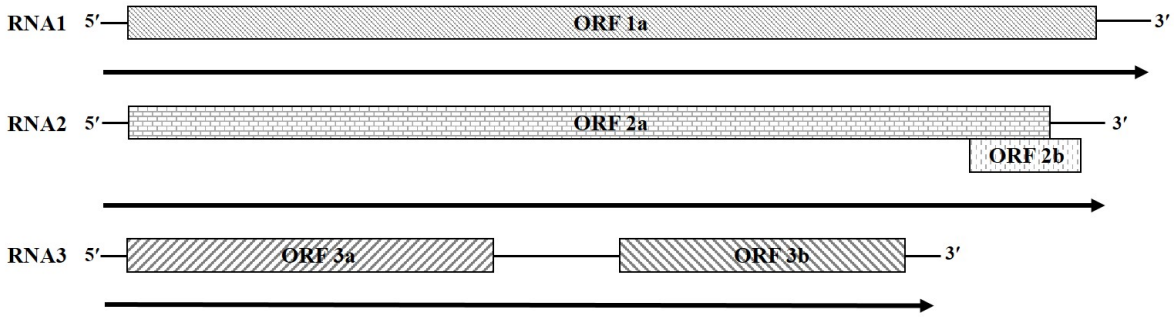

Fig. 2 A schematic representation showing the organization of the *cucumber mosaic virus* genome. Black arrows indicate the genomic regions of sequence contigs analyzed by high-throughput transcriptome RNA sequencing data

Table 1 Detailed information of *cucumber mosaic virus* (CMV) contigs derived from RNA sequencing

Target	Read count	FPKM*	Length (nt)	Query cover (%)	Identity (%)
CMV RNA1	1,933,455	31,020.81	3,269	100	94.62
CMV RNA2	1,804,283	32,306.03	2,950	100	98.07
CMV RNA3	2,871,859	71,451.71	2,179	100	98.21

* FPKM: Fragments per kilobase of transcript per million mapped reads.

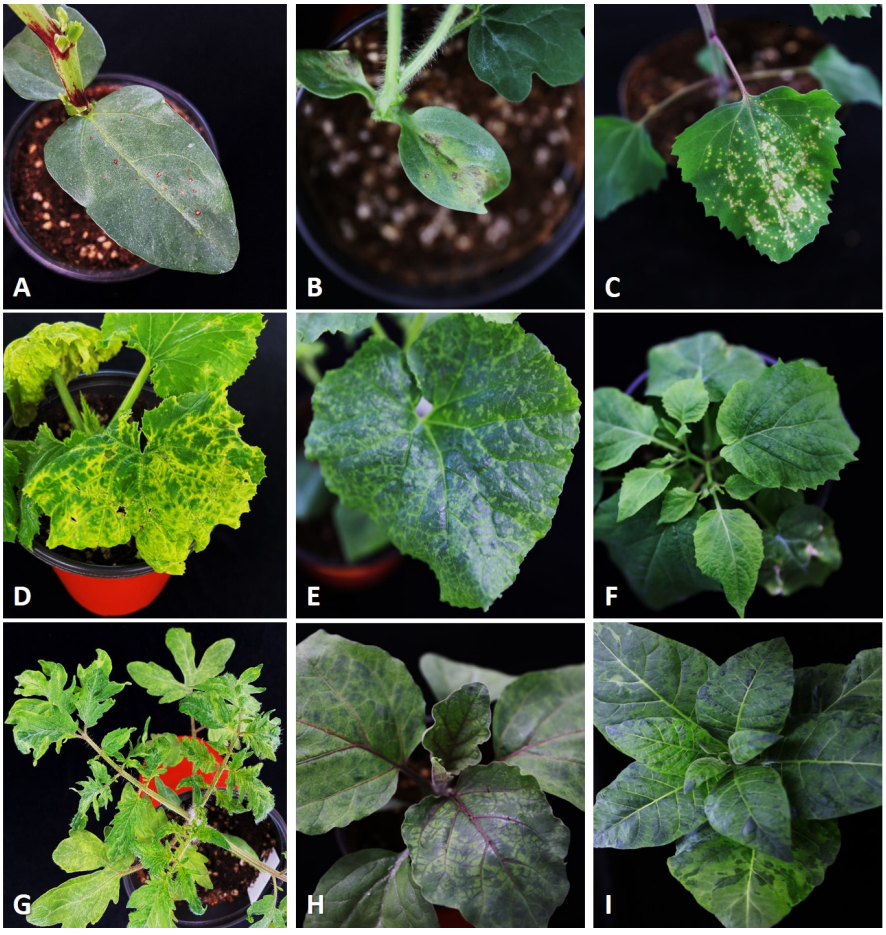

Fig. 3 Leaf symptoms displayed by indicator plants sap-inoculated with *cucumber mosaic virus* isolate BH (CMV-BH). Necrotic local lesions on *Vigna sinensis* (A), *Citrullus vulgaris* (B), and *Chenopodium amaranticolor* (C); (D) vein yellowing on leaves of *Cucurbita* spp.; mosaic on leaves of *Cucumis melo* (E) and *Physalis floridana* (F); (G) blistering, distortion, and mosaic on leaves of *Lycopersicon esculentum*; (H) mosaic on leaves of *Solanum melongena*; (I) blistering and mosaic on leaves of *Nicotiana tabacum* var. Samsun

Table 2 Symptoms observed in test plants that were mechanically sap-inoculated with the BH isolate of *cucumber mosaic virus* (CMV-BH)

Family	Species	Symptoms in the leaves	
		Inoculated	Upper
Cucurbitaceae	*Citrullus vulgaris*	NLL*	-
	Cucumis sativus	NLL	CS
	Cucurbita spp.	CLL	VY
	Cucumis melo	NLL	M
Leguminosae	*Vigna sinensis*	NLL	-
	Pisum sativum	NLL	-
	Phaseolus angularis	-	M
	Phaseolus vulgaris	-	Mo
	Glycine max	-	-
Solanaceae	*Lycopersicon esculentum*	-	B, M
	Nicotiana benthamiana	-	M
	Nicotiana debney	-	M
	Nicotiana glutinosa	-	M
	Nicotiana occidentalis	NLL	B, M
	Nicotiana rustica	-	B
	Nicotiana tabacum var. KY57	-	B, M
	Nicotiana tabacum var. Samsun	-	B, M
	Nicotiana tabacum var. Xanthi nc	-	M
	Nicotiana tabacum var. Turkish	-	B, M
	Physalis floridana	-	M
	Solanum melongena	-	M
Chenopodiaceae	*Chenopodium amaranticolor*	NLL	-

* B: Blistering, CLL: Chlorotic local lesions, CS: Chlorotic spots, M: Mosaic, Mo: Mottle, NLL: Necrotic local lesions, VY: Vein yellowing.

Table 3 Comparison of the nucleotide sequence identities of the complete coat protein gene between CMV-BH and other CMV isolates

Group	Subgroup IA									
Isolate	ZM	Fny	Y	Mf	RB	Leg	Pa	Z	Va	NT9
Identity (%)	99.2	98.8	98.8	98.2	98.8	97.6	95.9	97.1	96.3	95
Group	Subgroup IB			Subgroup II						
Isolate	CTL	Ix	IA	Ls	Trk7	Ly	Q			
Identity (%)	92.8	93	92.2	76.6	75.8	76.6	76.9			

develop any symptom on the upper leaves (Table 2). The mechanically sap-inoculated indicator plants were confirmed to be infected by PCR diagnosis, according to which, all indicator plants showing typical virus symptoms were confirmed positive to CMV. Among the five asymptomatic species, three (i.e., *C. vulgaris*, *V. sinensis*, and *G. max*) were actually positive, while two species (C. *amaranticolor* and *P. sativum*) were truly negative.

Phylogenetic analysis and homology comparison

To confirm the subgroup to which CMV-BH belongs, complete nucleotide sequence of the CP gene was determined using specific primer pairs. CMV-BH CP gene comprised 657 nucleotides (nt) encoding 218 amino acids. The CMV-BH sequence was deposited in the NCBI GenBank database under accession number LC11744. Phylogenetic analysis isolates at the nucleotide level divided 17 CMV isolates into three subgroups. CMV-BH belongs to subgroup IA and the closest isolate was ZM, isolated from *Zea mays* (JN180311) in Korea. While comparing sequence identities among 17 CMV isolates, CMV-BH was shown to share nt identity from 95% to 99.2% within subgroup IA, with isolates NT9 (D28780) and ZM (JN180311), respectively, and from 92.2% to 93% within subgroup IB, with isolates IA (AB042294) and Ix (U20219), respectively. Meanwhile, CMV-BH shared nt identities from 75.8% to 76.9% with isolates Trk7 (L15336) and Q (M21464), respectively (Table 3). Phylogenetic analysis and comparison

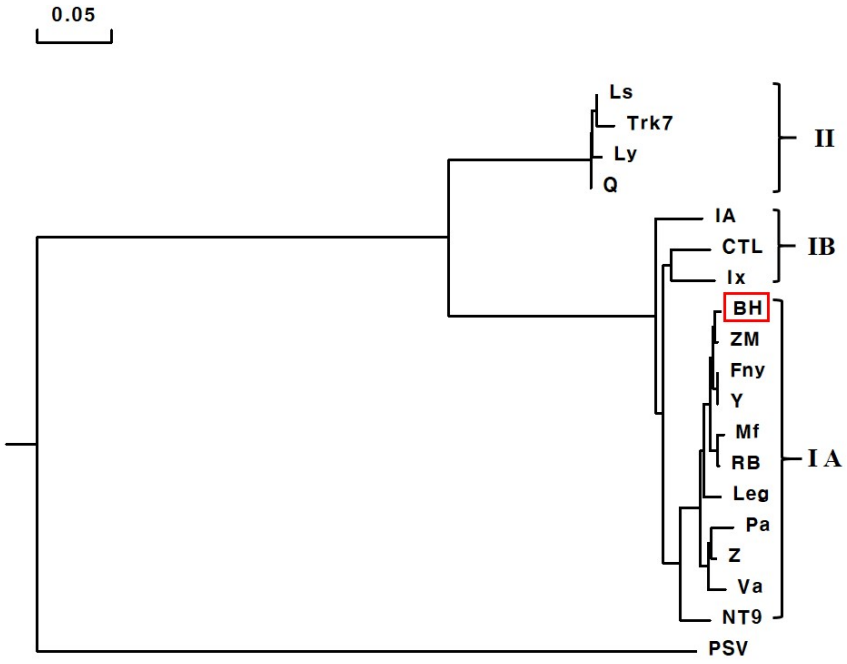

Fig. 4 Phylogenetic tree constructed for the complete coat protein gene of the CMV BH isolate and other CMV isolates using the Neighbor-Joining method. The bootstrap percentages are based on 1000 replications. *Peanut stunt virus* (PSV) was used as the outgroup. The NCBI GenBank accession number and the corresponding sequence information for virus isolates used in the analysis are as follows: BH (LC511744), ZM (JN180311), Z (GU327368), Y (D12499), Va (JX014248), Trk7 (L15336), RB (GU327365), Q (M21464), PSV (NC002040), Pa (AB290152), NT9 (D28780), Mf (AJ276481), Ly (AF198103), Ls (AF127976), Leg (D16405), Ix (U20219), IA (AB042294), Fny (D10538), and CTL (EF213025)

of nucleotide sequence identity proved that the CMV-BH isolate belongs to subgroup IA.

Discussion

In recent years, the advent of next generation sequencing (NGS) technologies has greatly facilitated virus research (Marston et al. 2013; Pecman et al. 2017). In Korea, NGS technology has been applied to detect and discover unreported and novel viruses from various plant species including, fruit trees, grain crops, weeds, etc. (Baek et al. 2019; Lim et al. 2015; Park et al. 2019; Yoo et al. 2015; Zhao et al. 2016). Nevertheless, studies of viral diseases on forest weeds are scarce at best. The identification of viral species by specific methods requires previous knowledge of the target virus or viruses we wish to test for. Due to the lack of virus infection information for many weed plants in Korea, specific methods [e.g. enzyme-linked immunoassay and PCR] are severely limited. To overcome this, we first applied NGS technology and then infection of indicator plants with the virus isolated was confirmed using PCR. The results of NGS indicated that nearly the complete genome of CMV was successfully obtained. In addition, the comparison of nucleotide sequences of amplicons by PCR, showed that there was almost no difference with respect to the obtained contig sequences using NGS. Therefore, it is proposed that NGS technology be applied first in order to efficiently identity weed viruses by nucleotide sequence comparison.

Over 1200 plant species are known to be infected by CMV, and various isolates/strains have been reported around the world (Edwardson and Christie 1997). A variety of symptoms of CMV is the joint result of the specific host plant species and the specific virus strain (Kaper and Waterworth 1981). Phylogenetic analysis and results of identity comparison indicated that the CMV-BH isolate showed highest similarity with the CMV-ZM (maize) isolate. Taking this into account, the bioassay results were compared. The comparison of the symptoms observed in the bioassay with CMV-ZM showed a large difference with respect to Leguminosae: while CMV-BH caused mosaic symptoms on the upper leaves in *Phaseolus angularis*, CMV-ZM did not infect this species. Furthermore, CMV-BH caused a systemic infection in *P. vulgaris*, whereas the ZM isolate caused only localized lesions. In addition, the symptoms on other indicator plants were similar to those caused by the ZM isolate (Kim et al. 2011). The difference in symptomatology within Leguminosae is likely due to the variety used and the experimental environment.

This is first report of CMV infecting *Pseudostellaria heterophylla* in Korea and the first biological and molecular

characterization of CMV isolates obtained from this plant species. Further studies are needed to elucidate the mechanism underlying seed-borne transmission, identify transmission vectors, document ecological characterization, and elucidate distribution patterns.

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